REMARKS

In view of the amendments and remarks presented herein, the Applicants request withdrawal of the rejections and favorable reconsideration of the claims.

I. Preliminary Remarks and Status of the Claims

Claims 71-115 are under consideration in the instant application. These claims stand variously rejected under 35 U.S.C. §112 first paragraph, for allegedly lacking enablement and/or written description, and under 35 U.S.C. §112, second paragraph as supposedly being indefinite for failing to particularly point out and distinctly claim the invention. The claims also were rejected under the judicially created doctrine of obviousness-type double patenting. Applicants respectfully traverse the rejections.

Claims 81-89 and 115 have been canceled herein by amendment, without prejudice or disclaimer. Claims 71 and 75 have been amended. Each of the amendments provided herein are fully supported by the specification as filed. Applicants reserve the right to pursue any canceled subject matter in additional continuation applications and the cancellation of the subject matter should not be viewed as abandonment or dedication to the public of such subject matter.

Claims 71-80 and 91-114 are presented for reconsideration in view of the present response, which Applicants believe places the claims in condition for allowance.

II. Request for Interview with Examiner Bunner

The undersigned representative thanks Examiner Bunner for the telephone conference on September 26, 2003 in which the instant response was discussed. Applicants respectfully submit that the instant response overcomes the outstanding rejections. However, if upon review of the above response, the Examiner feels that a further discussion would facilitate allowance of the claims, Applicants request an interview with the Examiner and, therefore, request that Examiner Bunner, at her convenience, contact the undersigned

representative .

III. Objections to the Specification

The Applicants thank the Examiner for holding the previously articulated objections to the specification in abeyance. Presented herein above are amendments to the specification which address the objections to the specification regarding sequence compliance, claimed priority, the Brief Description of the Drawings and references to other patent applications mentioned in the specification.

In the present amendment, Applicants have amended the specification so as to make reference to the sequence identifier information throughout the specification in order to bring the disclosure of particular nucleic acid and amino acid sequences in line with 37 C.F.R. §1.821(c).

Also by the foregoing amendment, the Applicants have updated and corrected the priority claim of the present continuing application. In addition, there are amendments to various individual pages of the specification, which amendments either correct obvious errors or are necessitated by the addition of the Sequence Listing enclosed herewith. Applicants also have amended the Brief Description of the Drawings to ensure that appropriate reference is made to Figures 24A-24B; Figures 29A-29B; Figures 30A-30B; Figures 42A, 42B, 42C, 42D, Figures 44A, 44B, 44C, and Figures 56A, 56B and 56D.

No new matter has been introduced by the amendments to the specification. In view of the amendments to the specification, Applicants respectfully request that the objections to the specification be withdrawn.

IV. Double Patenting Rejection.

Claims 71-115 were rejected under the doctrine of obviousness-type double patenting as being unpatentable over claims of U.S. Patent No. 6,204,363. Applicants thank the Examiner for holding the rejection in abeyance. Applicants will provide a terminal disclaimer should the instant claims indicated as allowable. In the event that the Examiner

wishes to discuss this point further, Applicants respectfully invite the Examiner to contact the undersigned representative.

V. Rejection under 35 U.S.C. §112, second paragraph should be withdrawn

The Examiner rejected claims 71-115 under 35 U.S.C. §112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. More particularly, the Examiner rejected the claims for using the term "an activity associated with SCF," indicating that this is a relative term and that allegedly does not apprise one of skill in the art of the scope of the invention. The Examiner stressed that it is inappropriate to read limitations from the specification into the claims. Initially, Applicants traverse the rejection because, while it is inappropriate to import limitations from the specification into the claims, terms used in the claims must nonetheless be read in the context in which they are used in the specification as a whole. One skilled in the art reading the claims in view of the specification would understand the term "activity associated with SCF" to mean a biological property of SCF. However, in order to further clarify the claim and expedite prosecution, Applicants have amended claim 71 to include the term "that enhances hematopoiesis," and remove the term "an activity associated with SCF," in accordance with the Examiner's suggestion at page 16 of the Office action. As all of the other claims ultimately depend from claim 71, the term also is incorporated into each of the other pending claims.

Applicants further clarify that those of skill in the art would understand that the compositions of the invention may enhance hematopoiesis *in vitro* and/or *in vivo*. Further those of skill in the art will understand that the composition of the present invention as claimed in claim 71 comprise a first portion that comprises SCF and a second portion that comprises the one or more cytokines. The SCF and the cytokine(s) may be formulated into a single discrete composition, or alternatively, be provided as separate and distinct components of the composition *e.g.*, in separate containers/receptacles.

In view of the foregoing amendment and comments, Applicants submit that the outstanding rejection of the claims under 35 U.S.C. §112, second paragraph is overcome.

Applicants request that the rejection be withdrawn and the claims be reconsidered for allowance.

VI. Rejection under 35 U.S.C. §112, first paragraph for lack of written description should be withdrawn

Claims 75 and 77-115 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which supposedly was not described in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention. Applicants respectfully disagree with the written description rejection and submit that the claims as amended are described in the specification in such a way as convey possession of the invention.

Claim 75 as amended her ein above recites:

> The composition of claim 73, wherein the SCF polypeptide is a polypeptide which comprises a polypeptide sequence selected from the group consisting of the amino acid sequence set out as 1-130, 1-133, 1-137, 1-141, 1-145, 1-148, 1-152, 1-156, 1-157, 1-158, 1-159, 1-160, 1-161, 1-163, 1-166, 1-168, 1-173, 1-178, 2-164, 2-165, 5-164, 11-164, 1-180, 1-183, 1-185, 1-188, 1-189, 1-220 and 1-248 as set out in Figures 42A-C, said polypeptide optionally consisting of an N-terminal methionine.

Claim 75 was amended herein above to remove the recitation of "1-100, 1-110, 1-120, 1-123, 1-127," and to correct the wording of the Markush language. As indicated by the Examiner at page 14, an SCF fragment that enhances hematopoiesis and comprises 1-130, 1-133, 1-137, 1-141, 1-145, 1-148, 1-152, 1-156, 1-157, 1-158, 1-159, 1-160, 1-161, 1-163, 1-166, 1-168, 1-173, 1-178, 2-164, 2-165, 5-164, 11-164, 1-180, 1-183, 1-185, 1-188, 1-189, 1-220 and 1-248 of SEQ ID NOs 46, 61 and 63 meet the written description provisions of 35 U.S.C. §112, first paragraph. Thus, claim 75 as amended meets the written description requirements and the rejection of claim 75 should be withdrawn.

The structural limitation of the sequence identity of the fragments is explicitly found in claim 75, and is therefore incorporated into each of dependent claims 77-80, and 91-

115. Claims 81-89 and 115 are canceled herein above thereby rendering any rejections thereof in the present application moot. The functional limitation that the fragments possess an activity that "enhances hematopoiesis" is incorporated into claim 75, (and therefore all the claims that depend from it,) because claim 75 ultimately depends from claim 71 which includes the feature that the SCF composition "enhances hematopoiesis." In light of the above comments and amendments, Applicants believe that the rejections of all of the pending claims under 35 U.S.C. § 112, first paragraph for lack of written description may now properly be withdrawn, and request reconsideration of the claims.

VII. Rejection under 35 U.S.C. §112, first paragraph for lack of enablement should be withdrawn

Claims 75 and 77-115 were rejected under 35 U.S.C. §112, first paragraph for assertedly lacking of enablement. There appear to be two grounds of rejection articulated by the Examiner under 35 U.S.C. §112, first paragraph: firstly, the Examiner objects to the size of the fragments encompassed by the claims, and secondly the Examiner objects to certain of the claims for reciting an intended use.

In the first rejection, which Applicants believe applies to claim 75 (and depend claims thereof) because this claim recites specific fragments, the Examiner's objection states that the specification provides enablement for "a composition comprising at least 130 amino acids of the amino acid sequence of SEQ ID NO:46, 61 and 63 that enhances hematopoiesis, [but] does not reasonably provide enablement for the SCF polypeptide consisting of the amino acid sequence as set out as 1-100, 1-110, 1-120, 1-123 and 1-127, as set out in Figures 42A-C and 44A-C or for an analog of SCF polypeptide of any of the sequences set forth in SEQ ID NO:46, 61 or 63 that possesses an activity associated with SCF." (Office page 3).

While Applicants have amended instant claims 75 to remove recitation of fragments "1-100, 1-110, 1-120, 1-123 and 1-127" and, therefore, have made the claims comport with subject matter that the Examiner admits is enabled by the specification, Applicants respectfully traverse the Examiner's reasons for rejecting the claims and reserve

the right to pursue the subject matter of compositions comprising "fragments of SCF shorter than 130 amino acids" in additional continuation applications.

Applicants respectfully submit that the Examiner's statement that the "fact patterns of *Ex parte Gasteambide, Thal, Rohracj, and Laroche* . . . are significantly different . . . [and] not binding . . . [because they teach] an effective process for making the claimed compounds from corresponding *known* steroids and describes method of using them for disclosed treatment of a condition." The Examiner's position that the facts of the instant case are different from those of *Ex parte Gasteambide* is based on the fact that the sequence of wild-type SCF was *previously unknown* and hence the skilled artisan "must resort to trial and error experimentation to generate the infinite number of analogs and SCF fragments smaller than 130 amino acids of SEQ ID NOs: 46, 61, or 63, as recited in the claims and screen them for a desired activity." The Applicants respectfully submit that this argument is unpersuasive and does not establish non-enablement of the claimed subject matter. The novel SCF of the invention is a cytokine. The sequence of this novel cytokine is provided in the specification, as are sequences of numerous fragments of the cytokine that enhance hematopoiesis. While the specific protein sequence of SCF may have been unknown at the time the instant application was filed, the fact that cytokines existed and that such cytokines enhance hematopoiesis was known to those of skill in the art. One of skill could take the SCF sequences of the invention, generate fragments and use them in known hematopoiesis assays to determine the effects of these fragments. This is not undue experimentation because it involves nothing more than mere routine screening of an activity in a known assay. The Examiner has not established that once the Applicants have identified the sequence of SCF it would have required undue experimentation to make the fragments and to use them to enhance hematopoiesis.

Moreover, the mere fact that some of the sequences have less activity than the full-length, or 1-164 size fragment of, SCF also does not establish non-enablement of the claimed invention as posited by the Examiner. As noted in the attached excerpt from Chapter 3 of a book entitled "Haemopoiesis: A Practical Approach" (*Eds.* Testa & Molineux, IRL Press, Oxford, U.K., 1993, see page 38) those of skill in the art have recognized that even if a

given hematopoietic growth factor alone exerts a marginal or modest stimulatory effect, when combined with CSFs such a factor will have a synergistic stimulatory effect on hematopoietic cells. This is further corroborated by the instant specification in Example 21 (pages 156-160, especially, page 160, lines 8-33), which specifically states that SCF has a synergistic effect when combined with other hematopoietic growth factors. Thus, so long as the SCF fragments possess some SCF activity, they are contemplated to be useful and within the scope of the claims.

Despite the above discussion which establishes enablement of fragments of SCF other than those that are at least 130 amino acids in length, however, Applicants' amendment to claim 75 obviates the grounds for rejection. While Applicants reserve the right to pursue broader subject matter in other applications and the amendments presented herein should not be considered as limiting any other such application, Applicants respectfully request that the instant rejection be withdrawn.

Applicants traverse the rejections articulated by the Examiner at section (v) of the Office action (pages 10-12). The Examiner indicated that the rejection was being applied to claims 81-90 (page 11, second paragraph.) The Examiner indicates that Applicants' previous arguments were not persuasive because the claims are being "interpreted as an intended use" in which "all possible epithelial cell disorders, stromal cell disorders, neural disorders, pigmentation disorder and germ cell disorders are to be treated." (page 11). Applicants have canceled claims 81-90 because the Examiner indicated that these claims were directed to an intended use. Applicants reserve the right to, and are, pursing various methods of use claims in other applications and do not view the cancellation of the instant claims 81-90 as an acquiescence to any of the Examiner's arguments with respect to methods or examples of the treatment of disease using the compositions of the present invention.

In view of the above discussion and amendments, Applicants respectfully request withdrawal of the rejection under §112, first paragraph, and reconsideration of the claims for allowance.

VIII. Conclusions

Applicants believe that all of the rejections have been overcome and the claims of the instant application are now in condition for allowance and request an early indication of such a favorable disposition of the case. The Examiner is invited to contact the undersigned with any questions, comments or suggestions relating to the referenced patent application.

Dated: September 30, 2003

Respectfully submitted,

By _____

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In vitro clonal assays for murine multipotential and lineage restricted myeloid progenitor cells

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1. Introduction

The first experiments describing the clonal growth of mouse haemopoietic progenitor cells immobilized in a soft-gel matrix *in vitro* were reported in the mid-sixties (1, 2). The significance of these observations was readily apparent to research workers in haematology and their pioneering studies allowed *quantification* of primitive progenitor cells that possess an ability to proliferate, differentiate, and develop into phenotypically and functionally mature myeloid cells. Indeed, this early work, along with the spleen colony-forming assays developed by Till and McCulloch in 1961 ((3) and Chapter 1), forms the basis of modern haematology and has culminated in the clinical use of the colony-stimulating factors.

In these early experiments, stimulation of haemopoietic progenitor (colony-forming) cells *in vitro* was achieved using a variety of feeder cells and medium conditioned by the growth of different tissues in culture: moreover, only colonies of granulocytes and/or macrophages were seen. Subsequently, however, colonies containing eosinophils, megakaryocytes, or erythroid cells, either alone or mixed with other myeloid cell lineages, were reported (4). There was then, of course, little appreciation of the diversity of haemopoietic cell colony-stimulating factors (CSFs) and, in retrospect, many of the controversies that existed in these early years (regarding the progenitor cells recruited under various culture conditions and the phenotypes of the mature cells produced) probably reflected the different types of CSFs to which the cells were being exposed. In the last few years, for example, the following haemopoietic cell growth factors have been molecularly cloned and purified to homogeneity: granulocyte–macrophage colony-stimulating factor (GM–CSF), granulocyte-CSF (G-CSF), macrophage-CSF (CSF-1, also known as M-CSF), interleukin-3 (IL-3, also called multi-CSF), and erythropoietin (Epo) (5). Each of these have *direct* stimulatory effects upon haemopoietic cells and show overlapping biological activities (see *Table 1*). In addition to

Table 1. Target cells for some purified haemopoietic cells growth factors and for conditioned media (CM)

	Mix-CFC	GM-CFC	M-CFC	G-CFC	Eos-CFC	Meg-CFC	BFU-E	CFU-E	Mast cells
IL-3	+	+	+	+	+	+	+	–	+
GM-CSF	–[a]	+	+	+	+	±	–[a]	–	–
CSF-1	–	+	+	?	–	–	–	–	–
G-CSF	–	–	–	+	–	–	–	–	–
Epo	–	–	–	–	–	±	–[a]	+	–
L-cell CM	–	+	+	–	–	–	–	–	–
WEHI-3B CM	+	+	+	+	+	+	+	–	+
Lung CM	–[a]	+	+	+	+	±	–[a]	–	–

[a] Modest effect on initial proliferation.

these CSFs, however, a range of other cytokines have been characterized that show no or very modest stimulatory effects upon haemopoietic progenitor cells when used alone, but which can exert powerful synergistic activities when used in combination with the CSFs. These include interleukins (IL) 1, IL-4, IL-5, IL-6, IL-11, and stem cell factor (SCF, also known as *kit* ligand or KL) (6, 7). Many of the CSFs and interleukins can themselves be produced by stromal cells, T lymphocytes, and/or macrophages present in normal bone marrow, it is important to be aware that some of the effects seen *in vitro* upon addition even of a single, defined, highly purified growth factor cannot necessarily be ascribed to a direct effect of this growth factor on the progenitor cells. It is for this reason that we often prefer to use haemopoietic cell populations that have been depleted of accessory cells and highly enriched for haemopoietic progenitor cells (see Chapter 2). Additionally, for some studies, we culture the cells under *serum-deprived* (serum-free) conditions in order to examine the potential effects that serum components may exert on the response of haemopoietic cells to the different growth factors.

Naturally, an ideal situation in many colony-forming assays is one where both the cell population being studied and the growth factor(s) being used are as homogeneous as possible. In many laboratories, however, this is difficult to achieve in view of the financial constraints (the cost of growth factors) and a lack of appropriate technology, (e.g. for fluorescence activated cell sorting) for enriching haemopoietic progenitor cells. In the absence of these, however, many important studies can still be performed using (fairly well defined) media conditioned by the growth of cell lines or tissues, and non-fractionated haemopoietic cells. Indeed, many of our routine experiments

employ such materials and a more reductionist approach (i.e. defined cells, serum-free cultures, pure growth factors) is linked only to more specific questions.

2. Reagents for clonal assays

2.1 Serum

The colony-forming cell (CFC) assays described below usually include a supplement of either horse serum (HS) or fetal calf serum (FCS). It is, however, critical that the batches of sera are pre-tested prior to bulk purchase: there can be an enormous variation in the plating efficiency and the differentiation of the CFC assays depending on the batch of serum used. Differences between serum batches suggests that there are marked variations in the levels of serum components capable of modulating colony formation. It is for this reason that *serum-deprived* media which are capable of supporting colony formation have been devised (see ref. 8 for review). Using the *serum-deprived* cultures described below it is possible to define the direct effects of certain cytokines on myeloid progenitor cell proliferation with a greater degree of certainty.

2.2 Preparation of *serum-deprived* culture medium

Serum-deprived culture medium is prepared by adding a variety of reagents to Iscove's modified Dulbecco's medium (IMDM). Often these reagents are unstable in solution and as such need to be made up fresh prior to use. *Table 2* lists the additives which are used to generate the complete *serum-deprived* medium.

Protocol 1. Preparation of the constituents for *serum-deprived* medium

1. Mix the following solutions in the proportions shown in *Table 2*. This table shows the volumes used for a final plating volume of 3.3 ml but obviously the volume can be adjusted as required.

   - IMDM
   - cell culture grade BSA[a] supplied as a 100 mg protein/ml solution in IMDM (Boehringer Mannheim 652 237), store at 4 °C for up to three months
   - soybean lipids supplied as a 2 mg/ml aqueous dispersion (Boehringer Mannheim 652 229), store at 4 °C for up to three months
   - cholesterol (Sigma C7402), dissolve in ethanol (7.8 mg/ml) and dilute 1 in 10 into BSA (100 mg/ml) immediately prior to use

Protocol 1. *Continued*

- linoleic acid (Sigma, L1376). Prepare a solution in absolute ethanol to a final concentration of 560 mg/ml and store at −20 °C for up to three months. Further dilute 1 in 100 with absolute ethanol and then dilute again (1 in 10) into the BSA (100 mg/ml) solution. Store for up to one week at 4 °C
- sodium pyruvate (Sigma, P2256), 100 mM, prepare in IMDM and store for up to one week at 4 °C
- glutamine 200 mM, sterile solution (Flow Laboratories), store at −20 °C
- α-thioglycerol (Sigma, M1753) 10 mM, prepare in IMDM and store for up to one week at 4 °C
- transferrin (30% iron saturated) is purchased as a 30 mg/ml sterile solution (Boehringer Mannheim 652 202), store at 4 °C for up to three months

2. Where the development of erythroid cells is desired add haemin to the cultures (9). Prepare bovine haemin chloride (Calbiochem 3741) as a 100 mg/ml solution in KOH (0.2 M). Dilute this solution 10:56.75 with IMDM to give a 20 mM stock solution, store at −20 °C. Add 33 µl of this solution and decrease the volume of IMDM accordingly (see *Table 2*).
3. Use in standard CFC assays (see *Protocol 5*[b,c]).

[a] BSA can also be prepared as specified in *Appendix 6*, but must be pre-tested prior to use.
[b] This protocol has been designed such that the volume of *serum-deprived* constituents (1 ml) replaced the volume of serum (0.66 ml) and BSA (0.33 ml) used in standard CFC assays (see *Protocol 5*).
[c] It is essential to use highly purified haemopoietic growth factors in these assays.

Table 2. Supplements for *serum-deprived* medium[a]

Reagent	Volume (µl)	Final concentration
BSA	330	10 mg/ml
Soybean lipids	42	25 µg/ml
Cholesterol	33	7.8 µg/ml
Linoleic acid	33	5.6 µg/ml
Sodium pyruvate	33	1 mM
Glutamine	33	2 mM
α-Thioglycerol	33	100 µM
Transferrin	33	300 µg/ml
IMDM	430	

[a] Calculated for a final volume of 3.3 ml of plating mixture—see *Protocol 1*.

3. Sources of growth factors

Purified, recombinant haemopoietic growth factors for CFC assays are now commercially available from several companies (see *Appendix 4*). However, the price of these products prohibits their use for routine work. It is therefore cost effective to prepare and test batches of conditioned medium (CM) from cell lines and tissues (such as mouse lung) which constitutively produce or can be induced to produce the myeloid colony-stimulating factors (albeit in an impure form). Examples of such cells are the WEHI-3B cells, a mouse myelomonocytic line which constitutively produces IL-3 (10) and L929 cells (L-cells) which produce CSF-1 (11). Another source of growth factors is an non-immunoglobulin producing myeloma cell line (X63 Ag8-653) transfected with and expressing one of several interleukin genes (IL-2, 3, 4, and 5) (12).

Mice treated with the bacterial cell surface component lipopolysaccharide exhibit symptoms similar to those seen in mice which have a bacterial infection. This leads to the production of haemopoietic growth factors from several tissues including the lungs. This can be exploited to obtain medium which contains haemopoietic growth factors, principally G-CSF and GM-CSF (13, 14) in the form of lung conditions medium (*Protocol 2*).

Protocol 2. Preparation of murine lung conditioned medium

- lipopolysaccharide (25 μg/ml in phosphate-buffered saline) from *S. typhosa* (Difco Labs, Detroit, Michigan, USA, 3124–25–6)
- 0.22 μm and 0.45 μm filters (Falcon 7111 and 7105)
- Fischer's medium (see *Appendix 6*)

1. Inject LPS solution intravenously into mice (200 μg/kg in 0.2 ml). See Section 2.4, Chapter 1 for method of intravenous injection.
2. Leave the mice for three hours.
3. Kill mice, remove the lungs and place one pair per glass McCartney bottle in 5 ml Fischer's medium.
4. Gas the bottles with 5% CO_2 in air and close the top firmly.
5. Incubate the bottles at 37 °C for two days.
6. Pour off the medium, taking care to leave the tissue residue behind. Centrifuge, (1000 *g* for 10 min) and filter the supernatant through a 0.45 μm and then a 0.22 μm filter. The 0.45 μm filter step is necessary if the CM is too thick to readily go through the 0.22 μm filter.
7. Store at 4 °C.
8. Test as described in *Protocols 4* and *5*.

Protocol 3. Preparation of cell line conditioned medium

A. *L929 cell conditioned medium*

- Dulbecco's modified Eagle's medium (DMEM, see *Appendix 6*) supplemented with 10% fetal calf serum (DMEM/FCS)
- T25 tissue culture flasks (Falcon 3013E)

1. Culture stock L929 cells in DMEM/FCS.
2. Sub-culture the stock cells into fresh medium once a week. Detach the cells by pipetting the growth medium directly onto the base of the flask. L929 cells are loosely adherent cells, which are easily detached. Add 1 ml cells to 9 ml fresh medium in a new flask (T25).
3. Harvest the growth medium from cells after seven days' growth following sub-culture at 1 in 10 dilution.

B. *Murine interleukin 3 conditioned medium (mIL-3)*

- DMEM supplemented with 5% FCS (DMEM/FCS 5%)

1. Culture the stock mIL-3 producer cells (12) in DMEM/FCS 5% supplemented with G418 (see Chapter 10), final concentration 1 mg/ml. *Do not add G418 when producing CM.*
2. Sub-culture the cells into fresh medium twice weekly at a dilution of 1 in 20 and 1 in 50.
3. Establish cells at between $2-4 \times 10^5$ cells/ml. Grow for two to three days and harvest conditioned medium.

C. *WEHI-3B cell conditioned medium*

1. Culture WEHI-3B cells in DMEM/FCS.
2. Sub-culture the cells into fresh medium twice weekly at a dilution of between 1 in 20 and 1 in 100.
3. Establish cells at approximately 5×10^4 cells/ml. Grow for five to seven days and harvest conditioned medium.
   (a) Maintain these cell lines at 37 °C in 5% CO_2 in air.
   (b) Like all factor-independent cells these cell lines are highly efficient at contaminating factor-dependent and slower growing cell lines by, e.g. aerosol droplets. Ensure they are kept separate.
   (c) Each batch of conditioned medium produced must be tested for activity, and because this is time consuming it is economical to produce large batches. Maintain the ratio of approximately 10 ml medium to 25 cm^2 growing surface (for economy routinely 50 ml/T75; 100 ml/T175).

(d) The figures given in *Protocol 3* for cell densities, dilutions for sub-culture, and incubation times for producing CM are a guide in that they are determined by the growth rate of the cells, which may vary depending on growth conditions. An important point is that CM should be harvested from cells at the top of the logarithmic phase of the growth curve, but before cells begin to die.

(e) The mIL-3-producing myeloma cell line produces no other colony-stimulating factors but we cannot exclude the possibility that other growth factors are in the conditioned medium.

Protocol 4. Harvesting and testing conditioned medium

1. Pour conditioned medium off the cells at the appropriate stage (*Protocol 3*). Pool together and mix the medium from the flasks that constitute the batch. CM may be stored at −20 °C if required, before proceding further.
2. Centrifuge (1000 *g*, 10 min) the conditioned medium and filter the supernatant through a 0.22 μm filter[a] (if the CM does not go through the filter readily an intermediate 0.45 μm filter may be used first).
3. Aliquot and store at −20 °C.
4. Test the CM in the GM-CFC assay (*Protocol 5*) to determine the optimal concentration for colony formation. Test the CM at a range of concentrations up to 25% (v/v).

[a] The purpose of the filter is to ensure removal of any residual cells but it is also a sterilizing filter so, if desired, the harvesting prior to this step need not be under aseptic conditions.

As a guide, the usual concentrations that the conditioned media are used at are 10–15% (lung-CM, L929 cell-CM, and WEHI-3B cell CM) and 1–5% (mIL-3 CM). Typically, dose–response curves for colony stimulation with CM show a maximal response which declines as the concentration of CM is increased further. This is probably due to increasing the dose of other components in the CM which may suppress colony formation. Obviously, this restricts the concentration of growth factor that can be achieved using CM as a source and, if high concentrations are required, recombinant or native purified growth factors may be necessary.

4. Preparation of cell populations

Various sources and types of haemopoietic cell populations can be used in CFC cell assays. These range from highly enriched cell populations, such as those described in Chapter 2, to preparations of bone marrow from mice

Table 3. Summary of colony-forming efficiencies of different cell populations in the Mix-CFC assay

Cell population (reference chapter)	**Colony-forming efficiency (%)**	**Final concentration cells/ml of culture**
Normal bone marrow (1)	0.1–0.24	$2–5 \times 10^4$
Highly enriched GM-CFC (2)	12–40	300–600
Enriched CFU-S population (2)	4–15	500–1000
Bone marrow from 5-fluorouracil treated mice (10)	0.01–0.02	5×10^5
FDCP-Mix cell line, e.g. A4 (6)	2–15	$2–8 \times 10^3$

treated with 5-fluorouracil (described in Chapter 10) which contain a heterogeneous population of cells of which only a small proportion have colony-forming potential. This means that the number of cells plated in each CFC assay to give a reasonable number of colonies is determined by the nature of the cell population. Prior to embarking on any programme of research concerning CFC assays it is essential to determine the number of cells plated which will give rise to between 30 and 120 colonies per plate. As an *approximate* guide the colony-forming efficiencies for some of the cell types described in this book are shown in *Table 3*. Cells are routinely used as soon as possible after isolation, however they may be left at 4 °C for up to four to five hours in medium plus 2% serum.

5. Colony-forming cell assays

Over the last twenty-five years many variations on the basic technique of the CFC assay have been developed which permit the identification of several distinct progenitor cell types. For example multipotent myeloid cells can be assayed using the Mix-CFC assay whilst primitive and more mature erythroid progenitor cells can be identified using the burst-forming unit–erythroid (BFU-E) and colony-forming unit–erythroid (CFU-E) assays respectively.

Although the methods below utilize either soft agar or methylcellulose as semi-solid support media for the CFC assays, it should be noted that methylcellulose is in some cases a more suitable medium, as is the case with BFU-E and CFU-E assays (see below). Also when the cells in the colonies are required for cytogenetic analysis methylcellulose is the preferred gelling agent (see Chapter 13).

5.1 Colony-forming cell–mix (Mix-CFC) assay

Most of the colony-forming assays using murine haemopoietic cell populations consist of variations on the basic *Protocol 5*.

Protocol 5. The colony-forming cell–mix (Mix-CFC) assay

- polystyrene tubes (Falcon 2003)
- Petri dishes (Falcon 3001, 35 mm diameter)
- P1000 Gilson Pipetman
- cell suspension (see Section 4)

1. In polystyrene tubes mix the constituents (as described in *Table 4*) in the order given. This gives a final volume of 3.3 ml which is sufficient material to generate triplicate plates for one Mix-CFC assay.
2. Melt the agar (3.3% w/v[a]) in a boiling water bath. Warm the rest of the plating mixture to 37 °C in a water bath.
3. Add 0.33 ml of agar to the plating mixture with a 1 ml disposable, sterile syringe (the same syringe can be used repeatedly provided it remains sterile). Mix thoroughly by repeated pipetting. It is better to add too little agar than too much.
4. Immediately plate out 1.0 ml of this mixture into each of three Petri dishes using a Pipetman.
5. Place dishes on a tray and allow them to set. If the ambient temperature is high then place the plates in a refrigerator at 4 °C for two to three minutes.[b]
6. Check that the agar has set, if so then place the tray in an humidified incubator gassed with 5% CO_2 and 5% O_2. These cultures are grown nominally at 37 °C, however this should not be exceeded and incubators are best set at 36 °C.
7. Incubate for between 7 and 12 days.
8. Count the number of individual colonies at the requisite time using a stereoscopic zoom microscope, (e.g. Olympus SZ) at about 25× magnification. Score day seven colonies with the Petri dish lid on so that the plate remains sterile for further incubation.

[a] This can be achieved by placing the bottle of agar in a beaker containing water which is heated by a Bunsen burner. The agar batches must be tested prior to use as some may give higher colony-forming efficiency than others, also the volume of agar solution required for optimal density in soft gel assays may vary from 8–12%, although most commonly 10% is best (see *Appendix 6*).

[b] If the Petri dishes are allowed to set at too slow a rate then the density of the gel will be uneven.

Table 4. Constituents of Mix-CFC assay

Additive	Volume (%)	Volume (ml) to make 3.3 ml
Fetal calf serum (pre-tested)[a]	20	0.66
BSA (10% v/v stock solution)[a]	10	0.33
IMDM	38	1.254
WEHI-3B cell conditioned medium (or other source of IL-3)	10	0.33
Epo (50 U/ml)	2	0.066
Cell suspension in IMDM (10 × final concentration required)	10	0.33

[a] Can be substituted for *serum-deprived* medium (*Table 2*).

(a) Where other additions are made the volume of IMDM should be adjusted so the final volume remains constant. Wherever possible additives should be dissolved in IMDM. If a solvent carrier such as dimethyl sulfoxide *has* to be added then appropriate control groups must be included in the experiment.

(b) In heavily used incubators an adequate level of humidity is essential to prevent the plates losing moisture. In this case open Petri dishes containing water placed on the tray with the cultures may help.

(c) If no hypobaric O_2 incubator is available colonies can be grown in 5% CO_2 in air but the colony-forming efficiency and erythroid cell maturation will be reduced.

(d) In *serum-deprived* cultures a greater concentration of haemopoietic growth factors is often required to achieve maximal levels of colony formation. Prior to embarking on any experiments using *serum-deprived* or serum-containing conditions it is necessary to establish the dose of the haemopoietic growth factor in question which gives optimal levels of colony formation.

(e) A colony is a group of > 50 cells, clusters containing between 10–50 cells may also be noted.

(f) Expect between 100 and 240 colonies per 10^5 bone marrow cells plated. Of these only about 10% will be mixed colonies containing erythroid cells.

(g) The assay allows the development of colonies containing mature cells from several distinct myeloid lineages, (e.g. granulocytes, monocytes, erythroid cells, and megakaryocytes). Colonies containing cells from only one or two of these lineages and/or primitive cells will also be present.

(h) Experience in identifying the types of colonies formed in the Mix-CFC assay can give the observer the skill to assess the types of colonies present

by their gross morphology. For an introduction to the discrimination between various types of colonies see the classical guide to colony-forming assays written by Metcalf (15). To confidently type the colonies, individual clones must be picked out for cytological examination (see *Appendix 2*).

5.2 Granulocyte–macrophage colony-forming cell (GM-CFC) assay

The GM-CFC assay is similar to the Mix-CFC assay described in *Protocol 5*. The differences in the assays are summarized as follows:

(a) Replace the fetal calf serum with the same volume of horse serum.

(b) Fischer's medium may be used instead of IMDM.

(c) Epo is omitted.

(d) The BSA added in *Protocol 5 can* be omitted from this protocol. However, the presence of the BSA has been shown to increase the linearity and reproducibility of the GM-CFC in an as yet undefined manner.

(e) The IL-3 can be replaced with other growth factors which also promote the formation of colonies from GM-CFC (see *Table 1*). These include CSF-1 (see *Protocol 3*). G-CSF (see *Protocol 2*), and GM-CSF (see *Protocol 2*). The specificities of these growth factors are shown in *Table 1*. *Appendix 4* lists the sources from which these purified recombinant haemopoietic growth factors can be obtained.

(f) Normally 100–240 colonies can be expected from 10^5 bone marrow cells plated.

(g) Colonies are routinely counted after seven days incubation. The granulocytic colonies can undergo lysis and effectively 'disappear' between day seven and day ten, presumably because of the short lifespan of mature granulocytes. Other colonies, developing from more primitive colony-forming cells may appear between day seven and day ten and in some experiments scoring colonies on both days is informative.

(h) In GM-CFC assays macrophage colonies, granulocyte/macrophage colonies, and granulocytic colonies are obtained. The macrophage colonies have a dispersed appearance, granulocyte/macrophage colonies have a compact centre consisting in large part of neutrophilic cells with a dispersed 'halo' of monocyte/macrophage cells, whilst granulocytic colonies (although fairly high in cell density) have a disordered appearance (4, 15). However, definitive typing of colonies can only be achieved by cytological examination (see *Appendix 2*).

(i) Different concentrations of the growth factors such as GM-CSF in the GM-CFC assay can markedly alter the type of colonies formed (i.e. the

proportions of granulocytic, macrophage, and granulocyte/macrophage colonies formed) (15). This underlines the necessity of always using optimal concentrations of growth factors.

5.3 Megakaryocyte colony-forming cell (Meg-CFC) assay

This assay is based on that described in *Protocol 5*.

(a) Follow steps **1** to **6** of *Protocol 5* to prepare *serum-deprived* growth medium. Add approximately 5% (v/v) mIL-3 CM instead of the 10% (v/v) WEHI-3B CM described in *Protocol 5*, make up the volume with IMDM. Incubate the assays for 11–14 days at 37 °C.

(b) An essential feature of this assay is the requirement for a staining procedure in order to identify colonies which contain megakaryocytes. Details of this staining procedure (based on the expression of the enzyme acetylcholinesterase) are listed in *Appendix 2*.

(c) Using normal bone marrow as a source of cells expect an incidence of at least one cell staining as positive for acetylcholinesterase in 10% of the colonies formed. Pure megakaryocytic colonies containing three cells have an incidence of 10–20 per 10^5 normal bone marrow cells.

(d) IL-6 may enhance megakaryocytic development in the Meg-CFC assay (16, 17).

5.4 Assays for erythroid progenitor cells

These assays are distinct from those described above in that they require methylcellulose as the support medium. Although erythroid development does occur in soft agar it is more efficient in methylcellulose.

5.4.1 Burst-forming unit–erythroid (BFU-E) assay

(a) The preparation of the methylcellulose is described in *Appendix 6*. The methylcellulose solution prepared is used as a 1.6 to 2.7% (w/v) stock solution in IMDM. Different batches of methylcellulose vary in viscosity and concentrations should be tested for each batch. (Further details of the handling use of methylcellulose are given in Chapter 5).

(b) The additives are the same as in *Protocol 5* with the following exception: the methylcellulose solution in IMDM is added to give 48% (v/v) of the final plating mixture. This is achieved by preparing the methylcellulose in IMDM instead of adding the IMDM normally made as described in *Protocol 5* and *Table 4*.

(c) BFU-E derived colonies are usually multicentric and are composed of late normoblasts and non-nucleated red cells. They become recognizable after five to six days in culture and are usually scored at day eight. At this stage they are composed of three to eight clusters which appear to have

'burst' from a common centre. These clusters are like CFU-E (see below) in that they are composed of small cells and are of a brick to bright red colour due to haemoglobinization; however, the size of the clusters in BFU-E assays is somewhat larger than seen in CFU-E assays. Burst size varies from a few hundred cells to about 10 000 and is very heterogeneous both in terms of the number of cells and also the number of clusters composing it.

(d) Bursts may be scored sequentially between eight and about 21 days of incubation. Longer incubation times detect colonies derived from earlier progenitor cells.

(e) Not infrequently bursts are not well haemoglobinized, they are however still recognizable by their characteristic morphology. It is advisable to check the accuracy of the scoring by picking out individual colonies for histological examination (*Appendix 2*).

(f) Expect an incidence of 20–100 bursts per 10^5 bone marrow cells. Note that the whole spectrum of myeloid colonies is also formed in these conditions.

(g) As the bursts are large colonies, adjust the initial inoculum to obtain 30–50 colonies per plate.

5.4.2 Colony-forming unit–erythroid (CFU-E) assay

The CFU-E assay, like the BFU-E assay, employs methylcellulose as a semi-solid support medium. In other respects the CFU-E assay can be based on the assay described in *Protocol 5* with two important exceptions.

- the source of IL-3 is *omitted* in the CFU-E assay and the volume made up with IMDM
- the Epo concentration is reduced to 0.2 U/ml

The sole haemopoietic growth factor added is erythropoietin (see *Table 1*). The CFU-E assay recognizes a more mature erythroid progenitor cell than the BFU-E assay, and there is a corresponding decrease in the size of the colonies and numbers of cells present in each colony.

(a) Murine CFU-E colonies are probably the most difficult to assess and score. They tend to be tight clusters of small cells each containing 6–60 cells which are late erythroid cells or non-nucleated red cells. The colonies appear to be dark rather than red and are best scored under 40–100 × magnification.

(b) CFU-E should be scored after two days incubation, the colonies disappear after three to four days due to the dispersion or lysis of the cells.

(c) Expect an incidence of 100 to 600 CFU-E per 10^5 murine bone marrow cells.

(d) At day three of the incubation groups of two to three adjacent clusters are probably derived from one erythroid progenitor cell, a 'late' BFU-E. If required these may be scored separately.

(e) Clusters of granulocyte or macrophages formed in CFU-E assays may be distinguished by the larger size of the cells. They also tend to be less tight.

6. Evaluation of clonogenic cells generated within colonies

The majority of cells comprising colonies which have developed from CFC in normal bone marrow are maturing and post-mitotic mature cells. However, a low incidence of clonogenic cells is also present and in certain experiments this incidence may be altered. The clonogenic cell content of colonies is measured by 'replating' the colonies, i.e. disaggregating them and plating in fresh semi-solid medium. The number of 'secondary' colonies that develop is a measure of the number of primitive clonogenic cells in the primary colony. Replating can be continued indefinitely at one to two week intervals as long as clonogenic cells persist but in normal bone marrow, plated under conditions described in this chapter, few secondary and no tertiary colonies are usually seen. This technique is particularly useful in experiments investigating the balance between self-renewal and differentiation of primitive cells, e.g. evaluating the comparative effects of growth factors, singly and in combinations, on primitive cells.

To determine the number of clonogenic cells per colony, individual colonies must be replated; often it provides sufficient information to replate a fraction of a whole plate which will give an average figure for clonogenic cells per colony. As the latter method involves less time and resources it is preferred unless data on individual colonies are required. The colonies can be plated from any of the CFC assays described in this chapter, in serum-containing or 'serum-deprived' conditions.

Protocol 6. Replating haemopoietic colonies

A. *Replating individual colonies*

1. Count the primary colonies.
2. Maintain sterility by placing the microscope in the microbiological safety cabinet. Pick individual colonies from the plate using a Pipetman P200 set at 100–200 µl.
3. Disaggregate the colony by aspirating it into 1 ml of plating mix.
4. Add 0.1 ml of agar, mix the suspension with a Pipetman P1000 and plate 1 ml in a 35 mm Petri dish.
5. Score the colonies after seven days.

B. *Replating whole plates*

1. Count the primary colonies.
2. Gently resuspend the 1 ml agar plate in IMDM using a P1000 to give a final volume of 3 ml and transfer to a tube. Be careful not to make air bubbles when resuspending the agar mix.
3. Add the desired fraction of the original plate to fresh plating mixture, add agar and plate into Petri dishes.
4. Score the colonies after seven days.

(a) Individual colonies derived from normal bone marrow usually produce secondary colonies in the range 0–15; however, occasionally up to 100 secondary colonies are obtained.

(b) The appropriate fraction of an agar culture to replate obviously depends on the number of secondary colonies that will develop. One-tenth of a 1 ml agar culture per secondary 1 ml culture is appropriate when the colonies are derived from normal bone marrow. This is equal to adding 1 ml of the suspended primary culture to a final plating volume of 3.3 ml and plating 1 ml secondary plates in triplicate. Secondary colonies in the range 0–5 per primary colony are usually obtained.

(c) The actual volume of the original agar mixture may be reduced to about 0.7 ml after seven days' incubation due to loss of moisture in the incubator. Initially it may take a bit of trial and error to add the correct amount of IMDM to get a final volume of 3 ml.

(d) When whole agar cultures are being replated the cell suspension already contains some agar so the volume of fresh agar added should be reduced to account for this (e.g. if the cells are added to give one-tenth of a primary plate per secondary culture the amount of agar added to the final volume of 3.3 ml should be reduced to 0.30 ml, see *Protocol 5*). Haemopoietic cell colony growth is severely reduced if the agar concentration exceeds 0.33%.

(e) Replating can be performed in the plating mixture of choice, but if the maximum expression of self-renewal of the clonogenic cells is desired horse serum, rather than fetal calf serum should be used (see Chapter 6, Section 4.1).

7. Concluding comments

The classification of clonogenic cells according to their growth potential is convenient for the purposes of this chapter, but in reality the range of clonogenic cells exist as overlapping populations rather than distinct and separate cell types. Furthermore, the CFC populations exist with a continuous

age structure so in the CFC assays we recognize clonogenic cells ranging from more to less immature rather than CFC of a truly homogeneous nature. This heterogeneity is reflected by the length of time colonies take to develop, i.e. the immature CFC take longer to form colonies than the more mature types.

With the exception of the more mature, lineage-restricted clonogenic cells (CFU-E and G-CFC) the mature cells which develop from the CFC are determined by both the potential of the CFC and by the growth factors that the clonogenic cells are exposed to. For example, a CFC plated in the Mix-CFC assay may produce a colony containing granulocytes, macrophages, and erythroid cells whereas if the same CFC is plated in the GM-CFC assay clearly only the first two potentials would be expressed. In other words, the development potential of a CFC as determined by plating in a CFC assay does not necessarily reveal the whole potential of that cell.

When clonogenic cells are plated in the presence of growth factors with a broad spectrum of target cells (*Table 1*) more than one class of CFC will be stimulated (unless the haemopoietic cell population is purified for one class of CFC). Thus, although Mix-CFC colonies develop in the Mix-CFC assay they will be accompanied by a predominance of GM-CFC colonies; likewise many GM-CFC colonies develop in the Meg-CFC assay and in the BFU-E assay. It is therefore important to distinguish the types of cells in the colonies. Normally this is done using vital stains or immuno-phenotyping.

The chapter is not an exhaustive list of clonogenic cell assays for haemopoietic progenitor cells and includes only the techniques in routine use in this department. Noteworthy omissions are;

(a) the blast cell colony assay developed by Ogawa (18) which detects a primitive cell type that takes two weeks to form colonies containing many primitive cells as measured by replating potential, and

(b) the HPP-CFC (high proliferative potential-CFC) (19) assay in which progenitor cells are stimulated with a combination of growth factors. These conditions produce very large colonies and some of the HPP-CFC are probably developmentally equivalent to the day 11 CFU-S (see Chapter 1).

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